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Self-Assembling Nanoparticle Conjugates

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional

Application No.: 60/528,407, filed on December 10, 2003, the contents of which

10 is incorporated by reference in its entirety.

TECHNICAL FIELD

This invention relates to magnetic nanoparticle conjugates and related

compositions and methods of use.

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RESEARCH

BACKGROUND

Non-invasive imaging of molecular expression in vivo with high

resolution and high sensitivity would be a useful tool in clinical diagnostics and

in biomedical research. Magnetic resonance imaging (MRI) offers certain well-

25 known advantages as a non-invasive imaging technology. For example, MRI

can potentially provide exceptionally high anatomic resolution approaching

single-cell levels (voxel of 20-40 μm^3). Moreover, recent innovations in

instrument design and contrast agent development indicate that even higher

resolution can be achieved non-invasively *in vivo*.

30 One application of nanotechnology in medicine is the development of

biocompatible nanomaterials as environmentally sensitive sensors and molecular

imaging agents. Preparations of magnetic particles designed for separation and

extraction use particles that are amenable to easy manipulation by weak applied

magnetic fields. These materials are typically micron sized and have a high

35 magnetic moment per particle; their effects on water relaxation rate are

5   unspecified and not relevant to their application. Nanoparticles do not respond

to the weak, magnetic fields of hand held magnets.. Thus, biocompatible

nanoparticles with unique optical and/or magnetic properties could have *in vitro*

and *in vivo* diagnostic applications. The ability to image specific enzyme

activities using such nanoparticles would have applications for detecting a

10   variety of diseases and evaluating targeted therapies in individual patients.

SUMMARY

This invention relates to magnetic nanoparticle conjugates and related

compositions and methods of use.

In one aspect this invention relates to compositions having at least two

15   nanoparticle conjugates, each nanoparticle conjugate having a magnetic

nanoparticle; and at least one substrate moiety, in which each substrate moiety is

linked to the nanoparticle and is chemically modified when the conjugate

interacts with a target enzyme. When the target enzyme is absent, the

nanoparticle conjugates are essentially monodisperse in liquids; and when the

20   target enzyme is present, the nanoparticle conjugates self-assemble into one or

more nanoparticle conjugate clusters through the formation of intermolecular

linkages between the chemically modified substrate moieties.

Embodiments can include one or more of the following features.

The conjugates can further include functional groups (e.g., amino, -

25   $NHC(O)(CH_2)_nC(O)$-, carboxy, or sulfhydryl groups, in which n is 0-100, e.g., n

can be 6) that link the nanoparticle to one or more substrate moieties.

The magnetic nanoparticles each can include a magnetic metal oxide

(e.g., a superparamagnetic metal oxide). The metal oxide can be iron oxide. In

some embodiments, the nanoparticles can be amino-derivatized cross-linked iron

30   oxide nanoparticles.

The substrate moieties can include a phenolic moiety, and can be

chemically modified by oxidation (e.g., one electron oxidation).

The target enzyme can be a protease or a peroxidase (e.g., a

myeloperoxidase or horseradish peroxidase).

35   Each of the monodisperse nanoparticle conjugates can have an average

particle size of between about 40 nm and about 60 nm. In some embodiments,

5   each of the monodisperse nanoparticle conjugates can have an average particle

size of about 50 nm.

Each of the nanoparticle conjugate clusters can have an average particle

size of between about 400 nm and about 500 nm. In some embodiments, each of

the nanoparticle conjugate clusters can have an average particle size of about

10   450 nm.

Each of the monodisperse nanoparticle conjugates can have an R1

relaxivity between about 5 and 30 mM^{-1} sec^{-1} and an R2 relaxivity between

about 15 and 100 mM^{-1} sec^{-1}.

The intermolecular linkages can be covalent linkages or non-covalent

15   linkages.

The formation of intermolecular linkages between the chemically

modified substrate moieties can be irreversible.

The formation of intermolecular linkages between the chemically

modified substrate moieties can result in crosslinking of the nanoparticle

20   conjugates.

The composition can further include a fluid media.

Self-assembly of the nanoparticle conjugates can result in the spin-spin

relaxation time of the fluid being decreased relative to the spin-spin relaxation

time of the fluid having essentially only monodisperse nanoparticle conjugates

25   present. The decrease in spin-spin relaxation time can be dependent upon the

concentration of the target enzyme.

The nanoparticle conjugate can have a formula X-(L)x-A, in which X is a

magnetic nanoparticle; L is -NH-, -NHC(O)(CH$_2$)$_n$C(O)-, -C(O)O-, or -SS-, in

which n is 0-20; A is substituted or unsubstituted aryl, substituted or

30   unsubstituted heteroaryl, substituted or unsubstituted aralkyl, substituted or

unsubstituted heteroaralkyl, substituted or unsubstituted aralkylamino, or

substituted or unsubstituted heteroaralkylamino; wherein substitutents are

selected from halo, hydroxy, C$_1$-C$_4$ alkoxy, or C$_1$-C$_4$ alkyl; and x is 0 or 1. X

can be magnetic metal oxide (e.g., iron oxide). x can be 1 and L can be -

35   NHC(O)(CH$_2$)$_n$C(O)- (e.g., n can be 6). A can be substituted aralkylamino, or

substituted heteroaralkylamino. In some embodiments, A is substituted with at

least one hydroxyl group, and A can be

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HO—
HO—〈benzene ring〉—CH₂CH₂NH∿∿∿

or

HO—〈indole ring〉—CH₂CH₂NH∿∿∿
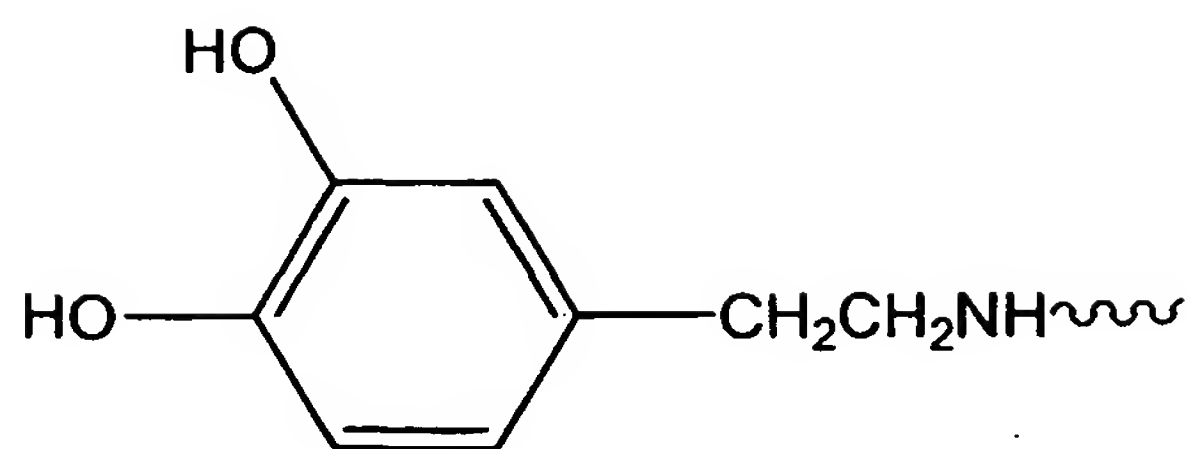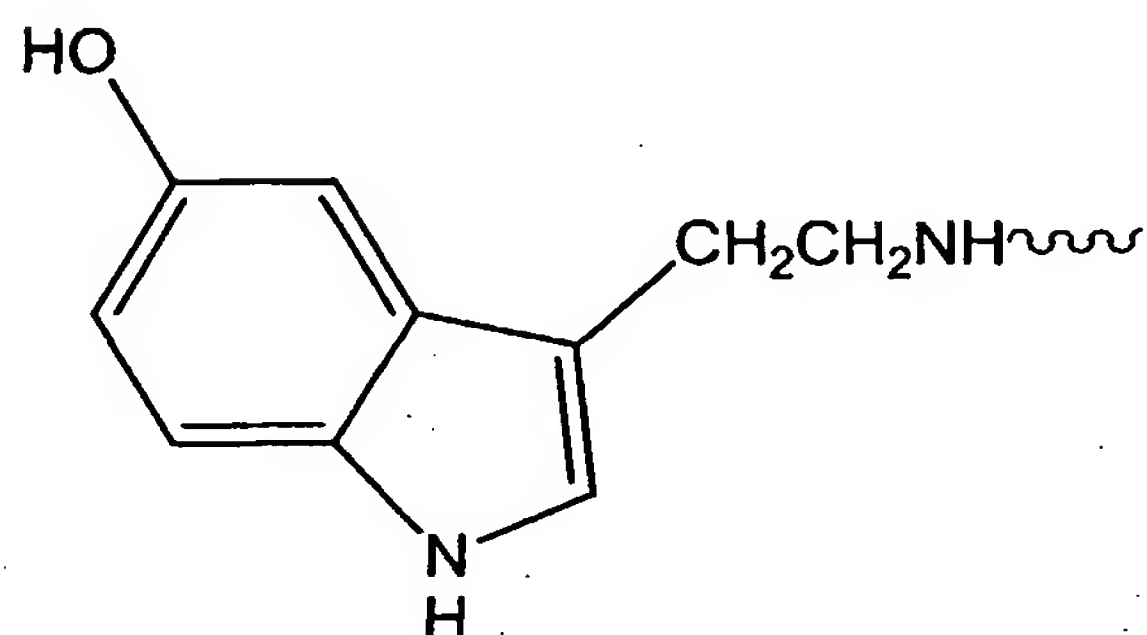
10   In some embodiments, the composition can include a population of at
least two nanoparticle conjugates, in which at least one nanoparticle conjugate
has a magnetic nanoparticle and/or substrate moiety that is different from the
magnetic nanoparticle and/or substrate moiety of one or more members in the
population. For example, a population can include one or more first nanoparticle
15   conjugates, each including a first magnetic nanoparticle and a first substrate
moiety, and one or more second nanoparticle conjugates, each including a
second magnetic nanoparticle and a second substrate moiety, whereby two types
of nanoparticle conjugates are present. The first and second magnetic
nanoparticles can be different and/or the first and second substrate moieties can
20   be different. The compositions can include a plurality of different types of
conjugates (e.g., 3, 4, 5, 6, 7, 8, 9, 10, 50, 90, 96, 100, 150, 200, 250, 300, 350,
360, 364, 400, or 500 types).

    In another aspect, this invention relates to *in vitro* methods for detecting
the presence of a target enzyme in a sample, the method includes (i) providing a
25   composition including at least two of the new nanoparticle conjugatesdescribed
herein; (ii) contacting the composition with a fluid sample; (iii) allowing time (a)
for the target enzyme to contact the nanoparticle conjugates and (b) for the

5   nanoparticle conjugates to self-assemble into clusters through the formation of
intermolecular linkages between the chemically modified substrate moieties; and
(iv) determining the spin-spin relaxation time of the fluid over time. A decrease
in spin-spin relaxation time indicates the presence of the target enzyme in the
sample.

10   In some embodiments, the methods further include the addition of
hydrogen peroxide or glucose oxidase.

In a further aspect, this invention relates to *in vivo* methods for detecting
the presence of a target enzyme in a subject (e.g., a human) by (i) administering
to the subject a composition including at least two of the new nanoparticle

15   conjugates described herein; (ii) allowing time (a) for the target enzyme to
contact the nanoparticle conjugates and (b) for the nanoparticle conjugates to
self-assemble into clusters through the formation of intermolecular linkages
between the chemically modified substrate moieties; and (iii) determining the
spin-spin relaxation time of the fluid over time. A decrease in spin-spin

20   relaxation time indicates the presence of the target enzyme in the subject.

The methods can further include the step of identifying the subject as
being in need of such detection.

In one aspect, this invention relates to the new self-assembling,
nanoparticle conjugates having a magnetic nanoparticle; and at least one

25   substrate moiety, in which each substrate moiety is linked to the nanoparticle and
is chemically modified when the conjugate interacts with a target enzyme.
When two or more nanoparticle conjugates are present and when the target
enzyme is absent, the nanoparticle conjugates are essentially monodisperse in a
liquid; and when two or more nanoparticle conjugates are present and when the

30   target enzyme is present, the nanoparticle conjugates self-assemble into one or
more nanoparticle conjugate clusters through the formation of intermolecular
linkages between the chemically modified substrate moieties.

In some embodiments, the conjugates can have a formula X-(L)x-A, in
which in which X is a magnetic nanoparticle; L is -NH-, -NHC(O)-, -

35   NHC(O)(CH$_2$)$_n$C(O)-, -C(O)O-, or -SS-, in which n is 0-20; A is substituted or
unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or
unsubstituted aralkyl, substituted or unsubstituted heteroaralkyl, substituted or
unsubstituted aralkylamino, or substituted or unsubstituted heteroaralkylamino;

wherein substitutents are selected from halo, hydroxy, C_1-C_4 alkoxy, or C_1-C_4 alkyl; and x is 0 or 1. X can be magnetic metal oxide (e.g., iron oxide). x can be 1 and L can be -NHC(O)(CH$_2$)$_n$C(O)- (e.g., n can be 6). A can be substituted aralkylamino, or substituted heteroaralkylamino. In some embodiments, A is substituted with at least one hydroxyl group, and A can be

or
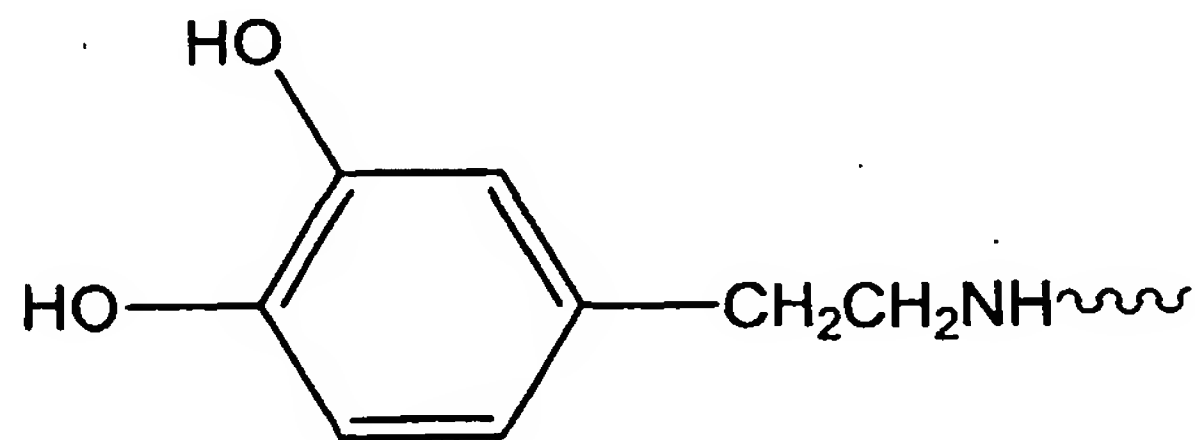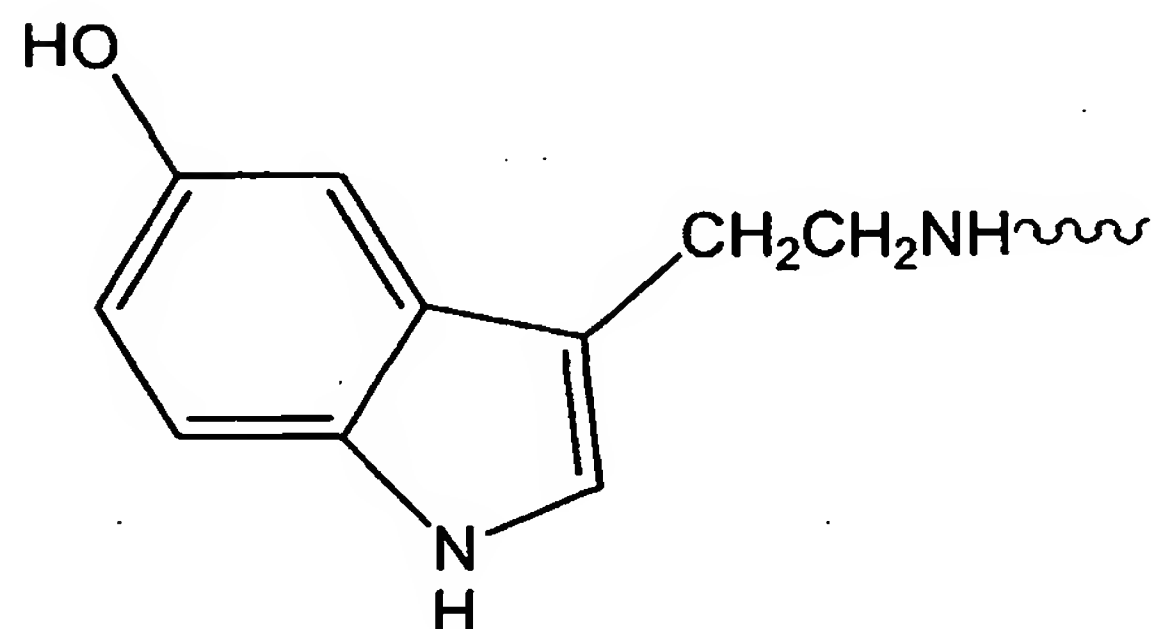
In another aspect, this invention relates to a packaged product including a composition having at least two of the new nanoparticle conjugates described herein.

Embodiments may include one or more of the following advantages.

In all embodiments, the nanoparticle conjugates are essentially monodispersed in the absence of a target enzyme, which can reduce the likelihood that the conjugates are cleared by the reticuloendothelial system prior to interaction with a target enzyme. Thus, the conjugates have relatively long circulation times *in vivo*.

In all embodiments, a single particle preparation is administered for imaging, which reduces the likelihood of observing multiple, differing pharmacokinetic profiles that can sometimes be associated with multi-particle preparations.

In some embodiments, the nanoparticle conjugates contain phenolic moieties as substrate moieties, in which relatively straightforward substitutions of the aromatic ring can result in incremental changes in the redox properties of the aromatic ring, thus allowing the substrate moieties to be readily tuned to different enzyme selectivities. Thus, a variety of target enzyme specific conjugates can be readily designed and prepared from the same basic nanoparticle scaffold.

In some embodiments, a single enzyme can result in the self-assembly of a plurality of nanoparticle conjugates, thereby achieving biological amplification at relatively low nanoparticle conjugate concentrations.

In some embodiments, preferential changes in R2 relaxivity can allow R1 relaxivity/R2 relaxivity magnetic resonance imaging to provide data that can be useful for measuring target enzyme concentrations.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a graphical representation of the particle size distribution by light scattering of the dopamine nanoparticle conjugates before incubation with horse radish peroxidase (HRP).

FIG. 1B is a graphical representation of the particle size distribution by light scattering of the dopamine nanoparticle conjugates after incubation with HRP.

5   FIG. 2 is a graphical representation of the effects of increasing HRP

concentration on the δT2 of a solution containing dopamine nanoparticle

conjugates with (solid squares) and without (solid triangles) hydrogen peroxide.

FIG. 3 is a graphical representation of the effects of increasing the

amount of sodium azide (inhibitor) on the δT2 of a solution containing dopamine

10   nanoparticle conjugates with hydrogen peroxide.

FIG. 4A is a graphical representation of δT2 values of the serotonin

nanoparticle conjugates in the presence of increasing amounts of

myeloperoxidase detected using a 1.5T clinical MRI both with (solid squares)

and without (solid triangles) hydrogen peroxide.

15   FIG. 4B is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: with peroxide;

0.0031 units/μL MPO.

FIG. 4C is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: with peroxide;

20   0.0061 units/μL MPO.

FIG. 4D is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: with peroxide;

0.0125 units/μL MPO.

FIG. 4E is a magnetic resonance image (1.5T MRI) corresponding to the

25   following myeloperoxidase activity data point shown in FIG. 4A: with peroxide;

0.025 units/μL MPO.

FIG. 4F is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: without

peroxide; 0.0031 units/μL MPO.

30   FIG. 4G is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: without

peroxide; 0.0061 units/μL MPO.

FIG. 4H is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: without

35   peroxide; 0.0125 units/μL MPO.

5   FIG. 4I is a magnetic resonance image (1.5T MRI) corresponding to the

following myeloperoxidase activity data point shown in FIG. 4A: without

peroxide; 0.025 units/µL MPO.

       FIG. 4J is a T2 (msec) magnetic resonance image signal intensity level

scale corresponding to the magnetic resonance images shown in FIGS. 4B-4I.

10   The levels shown in FIGS. 4B-4E occur in the top half of the scale, and the

levels of FIGS. 4F-4I occur in the bottom half of the scale. The level shown in

FIG. 4B occurs at about the top of the scale.

       FIG. 5A is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

15   (0 units/µL MPO) using dopamine-nanoparticle conjugates. There was

essentially no difference in signal intensity observed between this image and the

images shown in FIGS. 5B, 5C, and 5D.

       FIG. 5B is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

20   (0.0061 units/µL MPO) using dopamine-nanoparticle conjugates. There was

essentially no difference in signal intensity observed between this image and the

images shown in FIGS. 5A, 5C, and 5D.

       FIG. 5C is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

25   (0.025 units/µL MPO) using dopamine-nanoparticle conjugates. There was

essentially no difference in signal intensity observed between this image and the

images shown in FIGS. 5A, 5B, and 5D.

       FIG. 5D is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

30   (0 units/µL MPO) using serotonin-nanoparticle conjugates. There was

essentially no difference in signal intensity observed between this image and the

images shown in FIGS. 5A, 5B, and 5C.

       FIG. 5E is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

35   (0.0061 units/µL MPO) using serotonin-nanoparticle conjugates.

       FIG. 5F is a magnetic resonance image (1.5T MRI) of myeloperoxidase

activity

5   (0.025 units/μL MPO) using serotonin-nanoparticle conjugates.

FIG. 5G is a T2 (msec) magnetic resonance image signal intensity level

scale corresponding to the magnetic resonance images shown in FIGS. 5A-5F.

The levels shown in FIGS. 5A-5D occur at about the top of the scale, the level of

FIG. 5E occurs in the top half of the scale. The level shown in FIG. 5F occurs at

10   about the bottom of the scale.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

General

This invention relates to magnetic nanoparticle conjugates and related

15   compositions and methods of use. The nanoparticle conjugates generally include

a magnetic nanoparticle (circled "NP" in Scheme 1 below), that is linked to at

least one substrate moiety (circled "S" in Scheme 1 below). The nanoparticle

conjugates may optionally contain functional groups that link one or more

substrate moieties to the nanoparticle. The substrate moiety can be any chemical

20   group that can participate in an enzyme (e.g., a target enzyme)-mediated

chemical reaction. As such, one or more nanoparticle-bound substrate moieties

can be chemically modified (shaded circled "S" in Scheme 1 below) upon

interaction of the conjugates with the target enzyme (e.g., a peroxidase, a

protease). When the target enzyme interacts with a population of two or more

25   nanoparticle conjugates, the conjugates can self-assemble into nanoparticle

conjugate clusters through the formation of intermolecular (i.e., interconjugate)

linkages between the chemically modified substrate moieties. In the absence of

a target enzyme, the nanoparticle conjugates are essentially monodispersed (e.g.,

in solution or in a nonhomogenous fluid media).

30   Scheme 1
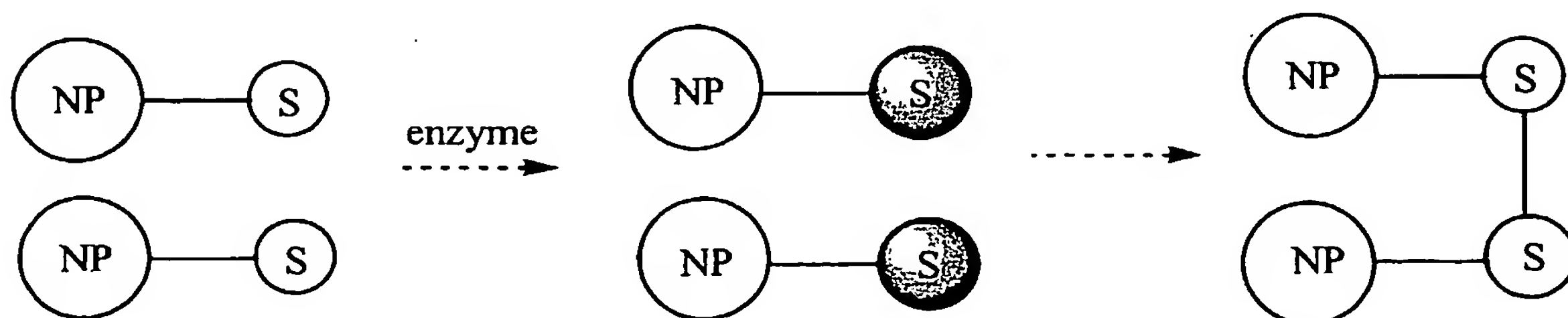
5   In general, the clusters formed from the nanoparticle conjugates described herein have one or more measurable properties (e.g., magnetic properties), that are altered, (e.g., increased or decreased) relative to the same one or more measurable properties of the monodispersed nanoparticle

10   conjugates. For example, the solvent (e.g., water) spin-spin relaxation times (T2) for solution phase nanoparticle conjugate clusters are relatively low in magnitude and differentiable, (e.g., by nuclear magnetic resonance (NMR) or magnetic resonance imaging (MRI)), from the relatively high solvent spin-spin relaxation times for the corresponding monodispersed, solution phase

15   nanoparticle conjugates. Accordingly, it is believed that solvent spin-spin relaxation times can be a useful parameter for determining the presence or absence of a target enzyme in biological samples containing nanoparticle conjugates with target enzyme-specific substrate moities. While not wishing to be bound by theory, it is believed that magnetic resonance amplification in the

20   form of a decrease in T2 would be observed in samples containing the target enzyme because interaction of the monodispersed nanoparticle conjugates (high T2) with the target enzyme results in the formation of one or more clusters (low T2), thereby decreasing the observed T2 of the sample.

25   *Definitions*

The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1~C_{12} alkyl indicates that the group may have from 1 to 12 (inclusive) carbon atoms in it. The term "aralkyl" refers to an alkyl moiety in which one or

30   more alkyl hydrogen atoms is replaced by an aryl group. Examples of "aralkyl" include benzyl, 2-phenylethyl, 3-phenylpropyl, 9-fluorenyl, benzhydryl, and trityl groups. The term "heteroaralkyl" refers to an alkyl moiety in which one or more alkyl hydrogen atoms is replaced by an heteroaryl group. Examples of "heteroaralkyl" include, e.g., tryptaminyl.

35   The terms "aralkylamino" and "diaralkylamino" refer to –NH(aralkyl) and –N(aralkyl)$_2$ radicals respectively. The terms "heteroaralkylamino" and "diheteroaralkylamino" refer to –NH(heteroaralkyl) and –N(heteroaralkyl)$_2$ radicals respectively The term "alkoxy" refers to an –O-alkyl radical.

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The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom capable of substitution can be substituted by a substituent.

The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, alkenyl, alkynyl, heterocyclyl, heterocycloalkenyl, cycloalkenyl, aryl, aralkyl, heteroaralkyl, heteroaryl, aralkylamino, diaralkylamino, heteroaralkylamino, or diheteroaralkylamino group at any atom of that group. Any atom can be substituted. Suitable substituents include, without limitation, alkyl (e.g., C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 straight or branched chain alkyl), cycloalkyl, haloalkyl (e.g., perfluoroalkyl such as CF_3), aryl, heteroaryl, aralkyl, heteroaralkyl, heterocyclyl, alkenyl, alkynyl, cycloalkenyl, heterocycloalkenyl, alkoxy, haloalkoxy (e.g., perfluoroalkoxy such as OCF_3), halo, hydroxy, carboxy, carboxylate, cyano, nitro, amino, alkyl amino, SO_3H, sulfate, phosphate, methylenedioxy (-O-CH_2-O- wherein oxygens are attached to vicinal atoms), ethylenedioxy, oxo, thioxo (e.g., C=S), imino (alkyl, aryl, aralkyl), $S(O)_n$alkyl (where n is 0-2), $S(O)_n$ aryl (where n is 0-2), $S(O)_n$ heteroaryl (where n is 0-2), $S(O)_n$ heterocyclyl (where n is 0-2), amine (mono-, di-, alkyl, cycloalkyl, aralkyl, heteroaralkyl, aryl, heteroaryl, and combinations thereof), ester (alkyl, aralkyl, heteroaralkyl, aryl, heteroaryl), amide (mono-, di-, alkyl, aralkyl, heteroaralkyl, aryl, heteroaryl, and combinations thereof), sulfonamide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof). In one aspect, the substituents on a group are independently any one single, or any subset of the aforementioned substituents. In another aspect, a substituent may itself be substituted with any one of the above substituents.

The term "halo" or "halogen" refers to any radical of fluorine, chlorine, bromine or iodine.

The term "alkylene" refers to a divalent alkyl, e.g., -CH_2-, -CH_2CH_2-, and –$CH_2CH_2CH_2$-.

The term "alkenyl" refers to a straight or branched hydrocarbon chain containing 2-12 carbon atoms and having one or more double bonds. Examples

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5   of alkenyl groups include, but are not limited to, allyl, propenyl, 2-butenyl, 3-hexenyl and 3-octenyl groups. One of the double bond carbons may optionally be the point of attachment of the alkenyl substituent. The term "alkynyl" refers to a straight or branched hydrocarbon chain containing 2-12 carbon atoms and characterized in having one or more triple bonds. Examples of alkynyl groups

10   include, but are not limited to, ethynyl, propargyl, and 3-hexynyl. One of the triple bond carbons may optionally be the point of attachment of the alkynyl substituent.

       The term "cycloalkyl" as employed herein includes saturated cyclic, bicyclic, tricyclic,or polycyclic hydrocarbon groups having 3 to 12 carbons. Any

15   ring atom can be substituted. The cycloalkyl groups can contain fused rings. Fused rings are rings that share a common carbon atom. Examples of cycloalkyl moieties include, but are not limited to, cyclopropyl, cyclohexyl, methylcyclohexyl, adamantyl, and norbornyl.

       The term "heterocyclyl" refers to a nonaromatic 3-10 membered

20   monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively). The heteroatom may optionally be the point of

25   attachment of the heterocyclyl substituent. Any ring atom can be substituted. The heterocyclyl groups can contain fused rings. Fused rings are rings that share a common carbon atom. Examples of heterocyclyl include, but are not limited to, tetrahydrofuranyl, tetrahydropyranyl, piperidinyl, morpholino, pyrrolinyl, pyrimidinyl, quinolinyl, and pyrrolidinyl.

30   The term "cycloalkenyl" refers to partially unsaturated, nonaromatic, cyclic, bicyclic, tricyclic, or polycyclic hydrocarbon groups having 5 to 12 carbons, preferably 5 to 8 carbons. The unsaturated carbon may optionally be the point of attachment of the cycloalkenyl substituent. Any ring atom can be substituted. The cycloalkenyl groups can contain fused rings. Fused rings are

35   rings that share a common carbon atom. Examples of cycloalkenyl moieties include, but are not limited to, cyclohexenyl, cyclohexadienyl, or norbornenyl.

       The term "heterocycloalkenyl" refers to a partially saturated, nonaromatic 5-10 membered monocyclic, 8-12 membered bicyclic, or 11-14

5   membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6
heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected
from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or
S if monocyclic, bicyclic, or tricyclic, respectively). The unsaturated carbon or
the heteroatom may optionally be the point of attachment of the

10   heterocycloalkenyl substituent. Any ring atom can be substituted. The
heterocycloalkenyl groups can contain fused rings. Fused rings are rings that
share a common carbon atom. Examples of heterocycloalkenyl include but are
not limited to tetrahydropyridyl and dihydropyranyl.

The term "oxo" refers to an oxygen atom, which forms a carbonyl when

15   attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or
sulfone when attached to sulfur.

The term "acyl" refers to an alkylcarbonyl, cycloalkylcarbonyl,
arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of
which may be further substituted by substituents.

20   The terms "aminocarbonyl," alkoxycarbonyl," hydrazinocarbonyl, and
hydroxyaminocarbonyl refer to the radicals $-C(O)NH_2$, $-C(O)O(alkyl)$, $-C(O)NH_2NH_2$, and
$-C(O)NH_2NH_2$, respectively.

The term "interacts" refers to any contact, reaction, or binding that occurs

25   between a nanoparticle conjugate and a target enxyme.

It is understood that the actual electronic structure of some chemical
entities cannot be adequately represented by only one canonical form (*i.e.* Lewis
structure). While not wishing to be bound by theory, the actual structure can
instead be some hybrid or weighted average of two or more canonical forms,

30   known collectively as resonance forms or structures. Resonance structures are
not discrete chemical entities and exist only on paper. They differ from one
another only in the placement or "localization" of the bonding and nonbonding
electrons for a particular chemical entity. It can be possible for one resonance
structure to contribute to a greater extent to the hybrid than the others. Thus, the

35   written and graphical descriptions of the embodiments of the present invention
are made in terms of what the art recognizes as being one or more of the
predominant resonance forms for a particular species.

5   *Structure of Nanoparticle Conjugates*

In all embodiments the nanoparticle component of the conjugate is a
magnetic nanoparticle, (e.g., magnetic metal oxide, such as superparamagnetic
iron oxide). The magnetic metal oxide can also comprise cobalt, magnesium,
zinc, or mixtures of these metals with iron. The term "magnetic" as used herein

10   means materials of high positive magnetic susceptibility such as
superparamagnetic compounds and magnetite, gamma ferric oxide, or metallic
iron. Preferred nanoparticles include those having a relatively high relaxivity,
i.e., strong effect on water relaxation.

In all embodiments, at least one substrate moiety is covalently linked to

15   the nanoparticle. In some embodiments, the substrate moiety is linked to the
nanoparticle *via* a functional group. The functional group can be chosen or
designed primarily on factors such as convenience of synthesis, lack of steric
hindrance, and biodegradation properties. Suitable functional groups may
include –NH-, -NHNH-, -O-, -S-, -SS-, -C(O)O-, -C(O)S-, -

20   $NHC(O)(CH_2)_nC(O)$-,
-NHC(O)-, -OC(O)$(CH_2)_n$(O)-, -OC(O)$(CH_2)_nC(O)$-, -C(O)$(CH_2)_nC(O)$-, -
$NH(CH_2)_nC(O)$-,
-O$(CH_2)_nC(O)$-, -S$(CH_2)_nC(O)$-, -NH$(CH_2)_n$-, -O$(CH_2)_n$-, or –S$(CH_2)_n$-, in which
n is 1-100 (e.g., x is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,

25   20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99).
Functional groups having cyclic, unsaturated, or cyclic unsaturated groups in
place of the linear and fully saturated alkylene linker portion, $(CH_2)_n$, may also
be used to attach substrate moieties to the nanoparticle. In some embodiments,
the functional group is –NHC(O)$(CH_2)_6C(O)$-. The functional group may be

30   present on a starting material or synthetic intermediate that is associated with
either the nanoparticle or the substrate moiety.

The number of substrate moieties linked to a nanoparticle may be
selected as desired. In some embodiments, a nanoparticle starting material can
contain one or more functional groups for attachment of substrate moieties, (e.g.,

35   2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, or 50 functional groups). The number of
substrate moieties that are ultimately linked to the nanoparticle can either be
equal to or less than the number of functional groups that are available for
attachment to the nanoparticle. In some embodiments, the number of substrate

5   moieties linked can correspond to a number that may be necessary to maintain

monodispersion of the conjugates in the absence of the target enzyme. In some

embodiments, the steric bulk of the substrate moiety or the nature of the enzyme

being targeted can also be determinative of the number of substrate moieties that

are ultimately loaded on to the nanoparticle. In any event, it is permissible for

10   the number of substrate moieties per nanoparticle conjugate to vary within a

given population of two or more nanoparticle conjugates.

The substrate moiety can generally be any chemical group that (1) can

function as a substrate for an enzyme (e.g., a target enzyme)-mediated (e.g.,

catalyzed) chemical reaction; and (2), when chemically modified, can form an

15   intermolecular linkage (e.g., a covalent or noncovalent linkage) with a second,

chemically modified substrate moiety. The substrate moiety can be a relatively

highly reactive substrate for the target enzyme, which readily undergoes

chemical modification upon interaction of the conjugate with the target enzyme.

In some embodiments, the substrate moiety is a substrate for a protease or a

20   peroxidase-mediated chemical reaction. In some embodiments, the target

enzyme-mediated reaction results in oxidation of the substrate moiety (e.g., a

one electron oxidation), to provide a radical as the chemically modified substrate

moiety.

In some embodiments, the substrate moiety is a phenolic moiety. As

25   used herein, "phenolic moiety" means a moiety containing a phenolic ring. As

used herein, a "phenolic ring" is a phenyl ring wherein at least one ring position

is substituted with a hydroxyl (OH) group, and other ring positions are

optionally substituted, provided that at least one ring position is unsubstituted

(see structures A and B below). In some embodiments, the phenyl ring may

30   further contain a fused heteroaryl ring (e.g., structure B).
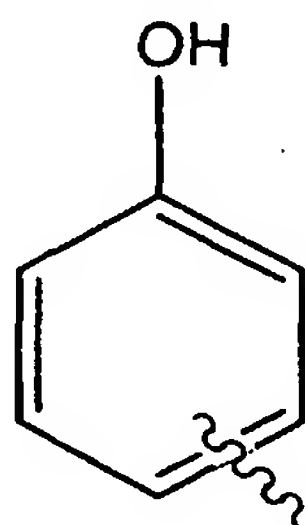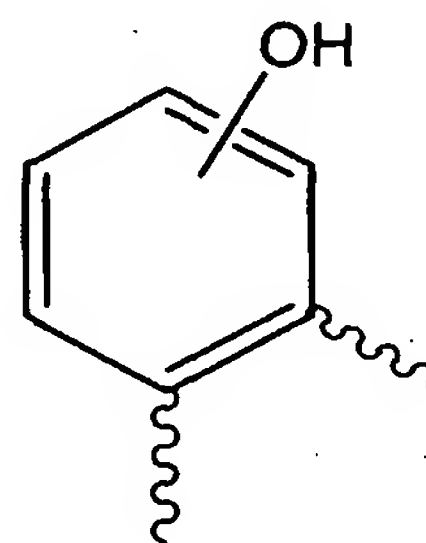
A   B

5

      Numerous structural variations are permissible in the phenolic moiety, and the phenolic moiety can be substituted with electron donating or withdrawing groups so as to alter the electronic properties (e.g., the redox properties), of the aromatic ring π-electon system. For example, the *ortho* and/or

10 *para* positions relative to the hydroxyl group can be substituted with OH, or C_1-C_4 alkoxy (e.g., OCH_3). When both *para* positions are substituted, the substituents can be the same or different. In another variation, an amino group or an amido group is substituted at a *meta* position on the phenolic ring. The effect(s) of the various substitutions possible on the phenolic ring can be

15 predicted by one of skill in the art according to known principles of organic chemistry, based on the identities of the substituents and their relative positions on the ring. See, e.g., L.G. Wade, Jr., 1988, *Organic Chemistry*, Prentice-Hall, Inc., Englewood Cliffs, NJ at 666-669. For example, an amino group at the *meta* position (relative to the hydroxyl group) is relatively strongly activating, i.e., this

20 substitution enhances the electron donor ability of the aromatic ring.

      Under certain conditions, phenolic moieties can function as electron donors in enzyme-catalyzed reductions (e.g., a peroxidase-catalyzed reduction of hydrogen peroxide). Oxidation, (e.g., one electron oxidation), of a phenolic moiety can provide a free radical intermediate, (e.g., a tyrosyl radical), which, in

25 turn, may couple with a second free radical intermediate, (e.g., a second tyrosyl radical), to form a covalent carbon-carbon single bond between the two radical intermediates (see Scheme 2 below). Carbon-carbon bond formation may occur in an intermolecular manner, resulting in, for example, cross-linking of the two phenolic moieties. One electron reduction of phenolic moieties and cross linking

30 of tyrosyl radicals are described in the art, (e.g., Heinecke, J. W. *Free Radic Biol Med* **2002**, *32*, 1090-1101; Heinecke, J. W., et al. *J Biol Chem* **1993**, *268*, 4069-4077; Winterbourn, C. C., et al. *Biochem Biophys Res Commun* **2003**, *305*, 729-736; McCormick, M. L., et al. *J Biol Chem* **1998**, *273*, 32030-32037).

5

Scheme 2
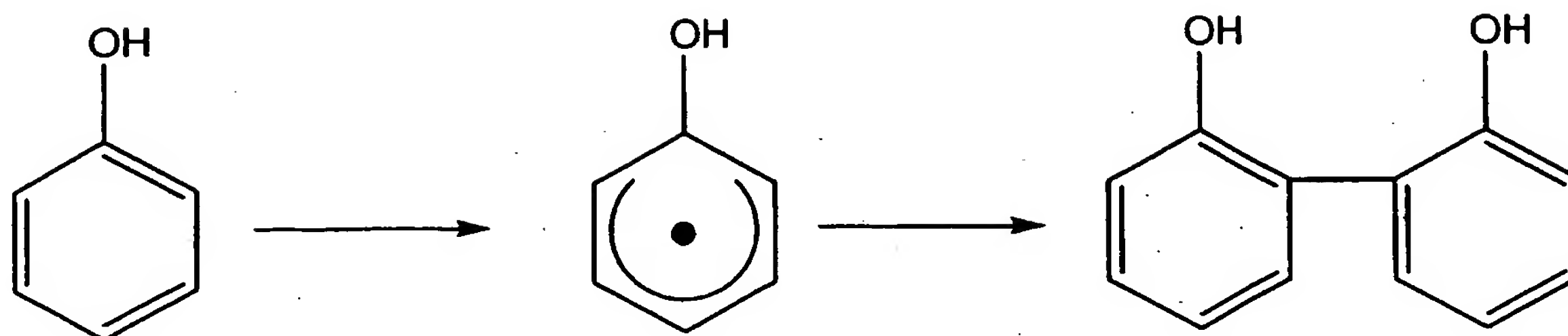
Accordingly, nanoparticle conjugates having phenolic substrate moities
10   (Structure **I** in Scheme 3 below) can be useful for detecting the presence of

target enzymes that mediate reductions, (e.g., peroxidases). While not wishing

to be bound by theory, it is hypothesized that interaction of **I** with such a target

enzyme would provide structure **II** (see Scheme 3 below) in which the substrate

moieties have been chemically modified to form free radicals *via* one electron

15   oxidation. The enzyme-induced formation of these radicals would then be

followed by result in subsequent intermolecular, *ortho, ortho* cross-linking

between the chemically modified phenolic substrate moieties to provide the self-

assembly **III**, (see Scheme 3 below), providing measurable changes in the

magnetic resonance signal.

5

<div align="center">Scheme 3</div>

(I)   (II)

(III)
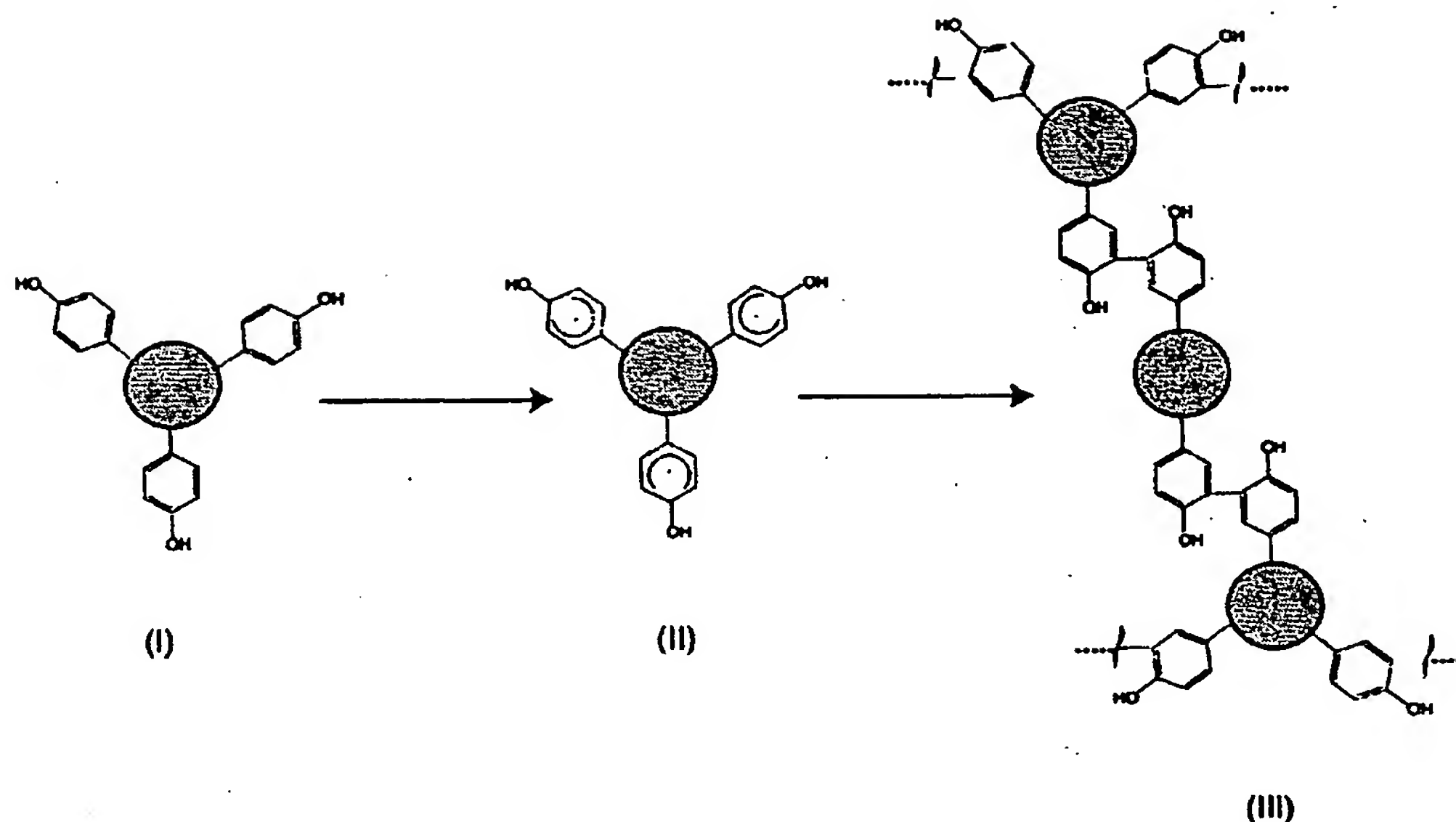
One subset of nanoparticle conjugates has a formula X-(L)x-A, in which:

X is a magnetic nanoparticle;

10   L is a functional group that may include –NH-, -NHC(O)-, -

NHC(O)(CH$_2$)$_n$C(O)-,

-C(O)O-, or –SS-, in which n is 0-20;

A is a substrate moity that may include substituted or unsubstituted aryl,

substituted or unsubstituted heteroaryl, substituted or unsubstituted aralkyl,

15   substituted or unsubstituted heteroaralkyl, substituted or unsubstituted

aralkylamino, or substituted or unsubstituted heteroaralkylamino; wherein

substitutents are selected from amino, halo, hydroxy, C$_1$-C$_4$ alkoxy, or C$_1$-C$_4$

alkyl; and x is 0 or 1.

A useful subset includes those conjugates in which X is an iron oxide

20   nanoparticle, x is 1, L is –NHC(O)(CH$_2$)$_6$C(O)-, and A is aralkylamino

substituted with at least one hydroxyl group, (e.g., Structure C in which the

substrate moiety is derived from dopamine) or heteroaralkylamino substituted

with at least one hydroxyl group (e.g., Structure D in which the substrate moiety

is derived from serotonin).

5

C

D
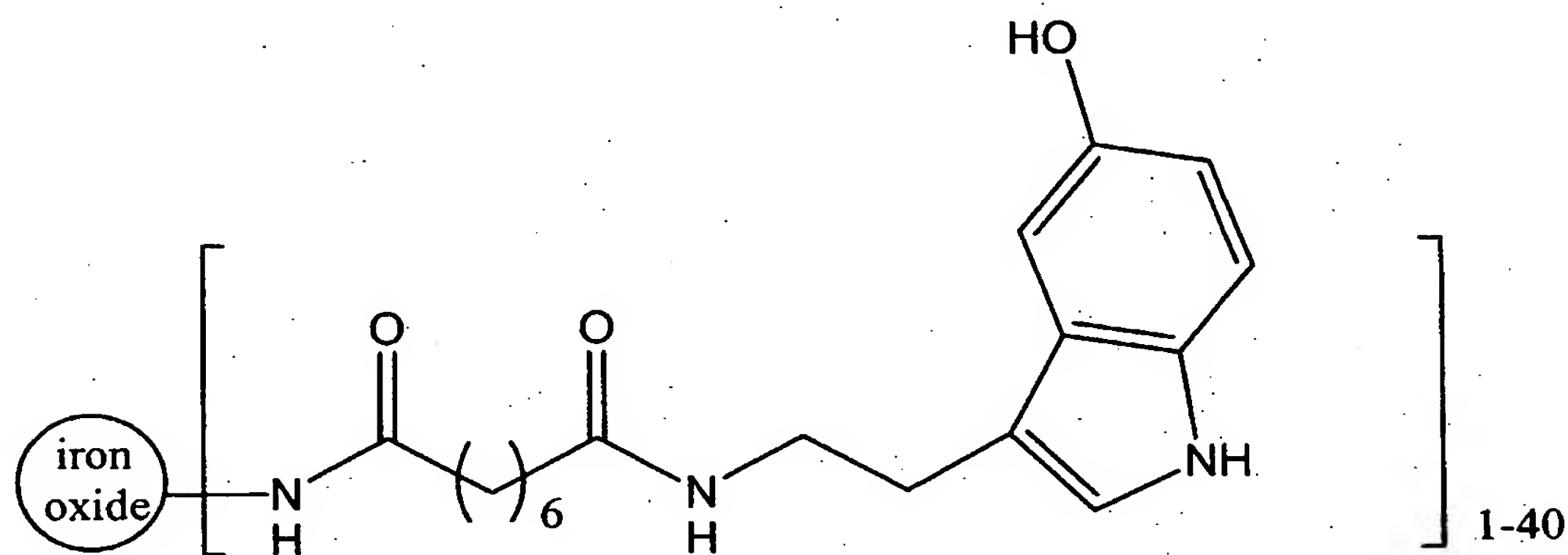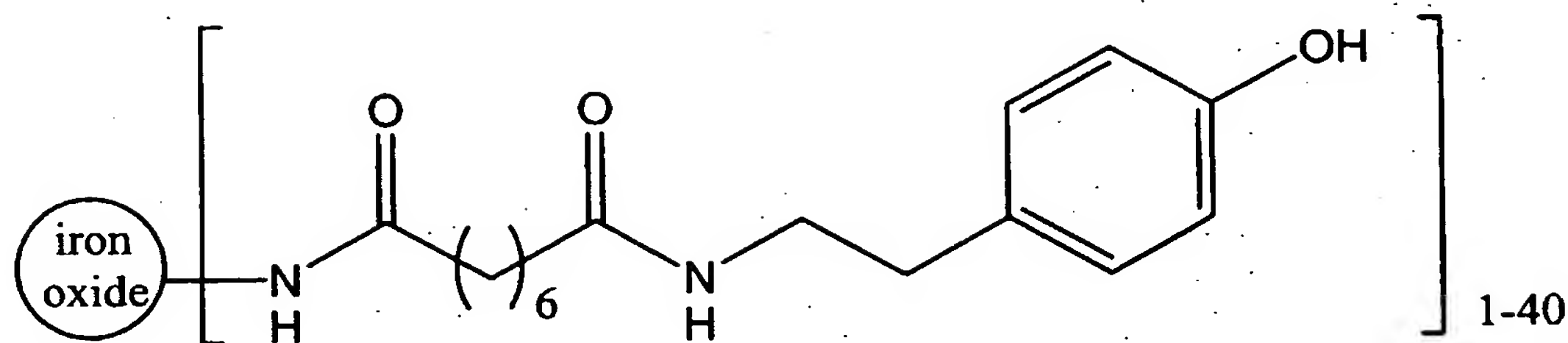
In general, the overall size of the nanoparticle conjugates is about 15 to 200 nm, e.g., about 20 to 100 nm, about 40 to 60 nm; or about 50 nm. The metal oxides are crystals of about 1-25 nm, e.g., about 3-10 nm, or about 5 nm in

10   diameter.

The conjugates have a relatively high relaxivity owing to the superparamagnetism of their iron or metal oxide. They have an R1 relaxivity between about 5 and 30 mM^{-1} sec^{-1}, e.g., 10, 15, 20, or 25 mM^{-1} sec^{-1}. They have an R2 relaxivity between about 15 and 100 mM^{-1} sec^{-1}, e.g., 25, 50, 75, or

15   90 mM^{-1} sec^{-1}. They typically have a ratio of R2 to R1 of between 1.5 and 4, e.g., 2, 2.5, or 3. They typically have an iron oxide content that is greater than about 10% of the total mass of the particle, e.g., greater than 15, 20, 25 or 30 percent.

20   *Synthesis of Nanoparticle Conjugates*

In some embodiments, nanoparticles having functional groups, (e.g., electrophilic functional groups such as carboxy groups or nucleophilic groups

5   such as amino groups) can be employed as starting materials for the nanoparticle conjugates.

Carboxy functionalized nanoparticles can be made, for example, according to the method of Gorman (see WO 00/61191). In this method, reduced carboxymethyl (CM) dextran is synthesized from commercial dextran.

10   The CM-dextran and iron salts are mixed together and are then neutralized with ammonium hydroxide. The resulting carboxy functionalized nanoparticles can be used for coupling amino functionalized groups, (e.g., a further segment of the functional group or the substrate moiety).

Carboxy-functionalized nanoparticles can also be made from

15   polysaccharide coated nanoparticles by reaction with bromo or chloroacetic acid in strong base to attach carboxyl groups. In addition, carboxy-functionalized particles can be made from amino-functionalized nanoparticles by converting amino to carboxy groups by the use of reagents such as succinic anhydride or maleic anhydride.

20   Nanoparticle size can be controlled by adjusting reaction conditions, for example, by using low temperature during the neutralization of iron salts with a base as described in U.S. Patent No. 5,262,176. Uniform particle size materials can also be made by fractionating the particles using centrifugation, ultrafiltration, or gel filtration, as described, for example in U.S. Patent No.

25   5,492,814.

Nanoparticles can also be synthesized according to the method of Molday (Molday, R.S. and D. MacKenzie, "*Immunospecific ferromagnetic iron-dextran reagents for the labeling and magnetic separation of cells,*" J. Immunol. Methods, 1982, 52(3):353-67, and treated with periodate to form aldehyde

30   groups. The aldehyde-containing nanoparticles can then be reacted with a diamine (e.g., ethylene diamine or hexanediamine), which will form a Schiff base, followed by reduction with sodium borohydride or sodium cyanoborohydride.

Dextran-coated nanoparticles can be made and cross-linked with

35   epichlorohydrin. The addition of ammonia will react with epoxy groups to generate amine groups, see Hogemann, D., et al., *Improvement of MRI probes to allow efficient detection of gene expression* Bioconjug. Chem. 2000. 11(6):941-6, and Josephson et al., "*High-efficiency intracellular magnetic labeling with*

novel superparamagnetic-Tat peptide conjugates," Bioconjug. Chem., 1999, 10(2):186-91. This material is known as cross-linked iron oxide or "CLIO" and when functionalized with amine is referred to as amine-CLIO or NH_2-CLIO.

Carboxy-functionalized nanoparticles can be converted to amino-functionalized magnetic particles by the use of water-soluble carbodiimides and diamines such as ethylene diamine or hexane diamine.

Compounds having structures corresponding to **C** and **D** were prepared using amino functionalized dextran-caged superparamagnetic iron oxide nanoparticles were used as the starting material. Dopamine or serotonin was conjugated to the aminated magnetic nanoparticles using suberic acid bis(N-hydroxysuccinimide ester) (DSS, Pierce Co). On average, each nanoparticle starting material had about 40 reactive amino groups, which were used for conjugation. Serotonin attachment was verified through its fluorescent emission at 345 nm. These nanoparticle conjugates were monodispersed in solution, having a narrow particle size distribution as determined by light scattering with an average particles size of about 50 nm. Particle size distribution for the dopamine-containing nanoparticle conjugates is shown in FIG. 1A. The water protons' spin-lattice relaxation (R1) of the nanoparticle conjugates was 25.8 s⁻¹mM⁻¹ while the spin-spin relaxation (R2) was 67 s⁻¹mM⁻¹. Relaxivity and size by light scattering can be determined by the methods described in, for example, Shen, T., et al. *Magn. Reson. Med. 29*, 599-604.

Uses of the Nanoparticle Conjugates

Solvent, (e.g., water), spin-spin relaxation times (T2) can be determined by relaxation measurements using a nuclear magnetic resonance benchtop relaxometer. In general, T2 relaxation time measurements can be carried out at 0.47 T and 40°C (Bruker NMR Minispec, Billerica, MA) using solutions with a total iron content of 10 µg Fe/mL.

Alternatively, T2 relaxation times can be determined by magnetic resonance imaging of 384-well plates (50 µL sample volume), allowing parallel measurements at higher throughput. In general, magnetic resonance imaging can be carried out using a 1.5 T superconducting magnet (Sigma 5.0; GE medical Systems, Milwaukee, WI) using T2-weighted spin echo sequences with variable echo times (TE = 25-1000 ms) and repetition times (TR) of 3,000 ms to cover

5   the spectrum of the anticipated T2 values. This technique is described in, for example, Perez, J. M., et al. *Nat Biotechnol* **2002**, *20*, 816-820; and Hogemann, D., et al. *Bioconjug Chem* **2002**, *13*, 116-121.

In some embodiments, the magnetic nanoparticle conjugates self-assemble in solution by the action of a specific peroxidase, with the enzyme-

10   mediated magnetic nanoparticle self-assembly acting as a magnetic resonance signal amplification system, which is sensitive to the enzymatic activity of the peroxidase. For an initial proof of the concept, we used horseradish peroxidase (HRP) an enzyme generally used in bioassays, while as a clinically relevant target, we used myeloperoxidase (MPO), an enzyme implicated in

15   atherosclerosis and inflammation (see, for example, Zhang, R., et al, *Jama* **2001**, *286*, 2136-2142; Brennan, M. L., et al. *N Engl J Med* **2003**, *349*, 1595-1604).

In the aforementioned experiments, dopamine and serotonin were selected and used as substrate moieties in two separates sets of nanoparticle conjugates (e.g., **C** and **D**) for detection of HRP and MPO, respectively. These

20   phenolic agents were thus chosen to be electron donors for the peroxidase-catalyzed reduction of hydrogen peroxide.

Generally, when a peroxidase is used *in vitro*, the new *in vitro* methods of the invention include providing a suitable amount of hydrogen peroxide in the tissue to be imaged. The hydrogen peroxide can be supplied directly.

25   Alternatively, it can be generated *in situ*, e.g., using glucose oxidase. If the hydrogen peroxide is enzymatically generated *in situ*, the generating enzyme can be administered directly (as a pre-formed enzyme) or can be expressed in the tissue from a suitable nucleic acid vector introduced into the tissue.

To test whether incubation of the nanoparticle conjugates with the

30   corresponding peroxidase would result in cluster formation, the dopamine-nanoparticle conjugates (10 µg Fe/mL, 0.1M phosphate pH 6.0) were incubated with HRP (0.9 units/µL) for 2 hours. After this incubation period, cluster formation was readily detectable by light scattering. The particle size distribution for the clusters are shown in FIG. 1B (particle size distribution

35   before incubation are shown in FIG. 1A). As expected, no cluster formation occurred in the absence of H_2O_2. These nanoclusters were stable in aqueous solution, did not continue growing in size and did not precipitate. Similar results

5   were observed when serotonin-nanoparticles were incubated with

myeloperoxidase.

Next, we investigated whether the peroxidase-mediated clustering would

result in T2 relaxation time changes (δT2) of the solution. For these

experiments, a solution of the HRP targeting nanoparticle conjugate (10 μg

10   Fe/mL, 0.1M phosphate pH 6.0) was incubated with different amounts of HRP

(0 – 0.9 Units/μL) for 2 hours at 4°C and the T2 relaxation times were measured

at 0.47T. Increasing δT2 values were observed upon incubation with increasing

amount of HRP, reaching saturation at a concentration of 0.9 units/μL in this

specific experiment as shown in FIG. 2. Essentially no changes in T2 were

15   observed in samples incubated with HRP in the absence of H_2O_2. To further

confirm that the detectable changes in T2 are caused by an HRP-mediated

mechanism, experiments were performed in which an increasing amount of

sodium azide, a known inhibitor of peroxidase, was added to the solution. As

expected, sodium azide inhibited HRP activity and reduced the δT2 changes in a

20   concentration dependent manner as shown in FIG. 3. T2 changes did not occur

in other control experiments using heat- or SDS-denatured HRP. The above

results confirm that the observed changes in δT2 were HRP-specific and that

these nonoparticle conjugates can be used as nanosensors for peroxidase activity

detection.

25   The ability of the nanoparticle conjugates to image myeloperoxidase

(MPO) activity was tested using a 1.5T clinical MRI imaging system. Recent

studies have demonstrated the importance of MPO in the development of

inflammation and cardiovascular diseases such as atherosclerosis and myocardial

infarction. High levels of intracellular MPO content has been found in plasma

30   samples from patients with coronary heart disease and acute coronary syndromes

while many other studies implicate MPO as one of the pathways for the

oxidation of low density lipoprotein in the artery wall (see, for example,

Heinecke, J. W. *Curr Opin Lipidol* **1997**, *8*, 268-274; Savenkova, M. L., et al. *J*

Biol Chem **1994**, *269*, 20394-20400; Leeuwenburgh, C., et al. *J Biol Chem* **1997**,

35   *272*, 3520-3526). It has also been observed that an increased number of MPO-

expressing macrophages can occur in eroded or ruptured plaques causing acute

5   coronary syndromes (see, for example, Sugiyama, S.; Okada, Y.; Sukhova, G.

K.; Virmani, R.; Heinecke, J. W.; Libby, P. *Am J Pathol* **2001**, *158*, 879-891).

A serotonin-containing nanoparticle conjugate (prepared as described

herein) was selected for the MPO imaging experiments because serotonin has

been reported to be a superior substrate for myeloperoxidase relative to

10   dopamine (see, for example, Allegra, M., et al. *Biochem Biophys Res Commun*

2001, *282*, 380-386; Dunford, H. B.; Hsuanyu, Y. *Biochem Cell Biol* **1999**, *77*,

449-457). The serotonin-nanoparticles ($3 \mu g$ Fe/mL, 0.1M phosphate pH 6.0)

were incubated with various amounts of myeloperoxidase both with and without

H_2O_2 in a 384 well-plate and imaged by MRI. Similar to the experiments

15   conducted with HRP, $\delta T2$ increased as a function of MPO concentration as

shown in FIG. 4A. Furthermore, we were able to demonstrate that $\delta T2$ changes

were of significant magnitude to be detectable using a clinical MR imaging

system (see FIGS. 4B-4E and 4J). Control samples consisting of MPO-

nanosensor incubated with myeloperoxidase in the absence of H_2O_2 showed no

20   significant increase in $\delta T2$ as expected (see FIGS. 4F-4I and 4J). Likewise, as

shown in FIGS. 5A-5G, the dopamine-containing nanoparticle conjugates did

not show any $\delta T2$ in the presence of MPO (i.e., essentially no difference in

signal intensity observed when dopamine-nanoparticle conjugates are incubated

with myeloperoxidase). The findings demonstrate that the selectivity of the

25   particle-bound substrate moieties is about the same as that for the particle free

substrates.

Substrate moieties are not limited to chemical groups that are substrates

for enzyme-mediated oxidation-reduction reactions. Many enzymes known in

the art, (e.g., polymerases), catalyze the formation of chemical bonds via

30   different reaction mechanisms.

In magnetic resonance (MR) imaging applications, the nanoparticle

conjugates can be used in methods for the detection and a spatial localization of

target enzymes in living systems. This is based, in part, on the ability of the

magnetic conjugates to effect water relaxation in media that generally will not

35   permit assays using light-based methods. Hence, the conjugates can function as

MR contrast agents or magnetic nanosensors for the detection of target enzymes

in vivo.

5   The new conjugates are essentially nontoxic to mammalian cells. The nanoparticle conjugates can be administered to a subject, e.g., a human or animal, such as a mammal (e.g., dogs, cats, cows, pigs, and horses). Various routes of administration known in the art can be used to achieve systemic or local delivery (e.g., orally, parenterally, by inhalation spray, topically, rectally,

10   nasally, buccally, vaginally or via an implanted reservoir). Compositions containing the nanoparticle conjugates of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles (e.g., a fluid media).

       Also within the scope of this invention is a method of screening substrate

15   moieties for selectivity for one or more target enzymes. For example, libraries of phenolic substrates attached to nanoparticles can be screened by high throughput NMR methods described herein (e.g., for numerous peroxidases).

OTHER EMBODIMENTS

20   A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.